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6. AUTHOR(S)

Robert R. H. Anholt, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Duke University Medical Center
Department of Neurobiology
Durham, NC 27710

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13. ABSTRACT (Maximum 200 words)

Our studies on the functional characterization of odorant receptors have developed in two directions. One direction is concerned with the characterization of the ligand specificity of a single defined odorant binding domain. The fourth and fifth transmembrane domains and their extracellular linker of the human β_2-adrenergic receptor have been replaced by the equivalent regions of odorant receptor I-15 (Buck and Axel, 1991), thus forming a chimaeric seven transmembrane domain containing G-protein linked receptor that couples a novel odorant binding domain consisting of the I-15 moiety (and perhaps part of the adrenaline binding site of the adrenergic receptor) to the intracellular regions of the β_2-adrenergic receptor that couples ligand binding to the production of cyclic AMP. Expression of this chimaera in eukaryotic cells and screening the expressed construct with an array of odorants is expected to identify a set of odorants that interacts with the ligand binding domain of the synthetic receptor. The binding site can then be characterized pharmacologically and the interactions of odorants with specific amino acid residues in the hybrid receptor can be studied. This information can then be used to characterize the odorant specificity of the parental I-15 receptor, that may not be identical, but is likely to be closely related to that of the chimaera. The second direction of our research program is concerned with the question of how an ensemble of odorant receptors with overlapping ligand specificities interacts to recognize a given odorant. To the best of our knowledge only one experimental system is available to address this issue directly: highly inbred lines of *Drosophila melanogaster* that contain single *P*-element inserts giving rise to mutations at single loci within a co-isogenic genetic background. This system allows a multidisciplinary approach that enables correlation of the expression of identified olfactory genes with behavior of the animal.

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FUNCTIONAL CHARACTERIZATION OF ODORANT RECEPTORS

FINAL REPORT

ROBERT R. H. ANHOLT

FEBRUARY 1, 1994

DAAL03-92-G-0390

DUKE UNIVERSITY MEDICAL CENTER

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1. FOREWORD

This progress report covers the period from September 1, 1992 through November 30, 1993. The Principal Investigator transferred his laboratory from Duke University to North Carolina State University effective January 1, 1994. He requested the project sponsored by ARO grant DAAL03-92-G-0390 to be terminated at Duke University on November 30, 1993 and to be continued as outlined in the original grant proposal under a new grant number at North Carolina State University. This final report covers progress made during the first year of this research project at Duke University.

2. TABLE OF CONTENTS

3. LIST OF ILLUSTRATIONS AND TABLES

4. PROGRESS REPORT

A. STATEMENT OF THE PROBLEM STUDIED

In recent years rapid progress has been made in elucidating the molecular mechanisms of olfaction (reviewed by Anholt, 1993). In vertebrates, odorants partition into the mucus that lines the chemosensory surface, a process facilitated through interactions with odorant binding proteins (Bignetti *et al.*, 1985; Pevsner *et al.*, 1985 and 1988; Schofield, 1988). Following their partition into and transport through the mucus, binding of odorants to receptors on the chemosensory membranes of dendritic cilia of olfactory receptor neurons (Buck and Axel, 1991; Levy *et al.*, 1991; Ngai *et al.*, 1993 a and b; Raming *et al.*, 1993) triggers transduction cascades accompanied by the formation of cyclic AMP (Pace *et al.*, 1985; Sklar *et al.*, 1986; Shirley *et al.*, 1986; Breer *et al.*, 1990) and/or inositol triphosphate (Huque and Bruch, 1986; Boekhoff *et al.*, 1990; Raming *et al.*, 1993) as second messengers. Subsequent opening of cyclic nucleotide-activated cation channels (Nakamura and Gold, 1987; Dhallan *et al.*, 1990; Firestein et al., 1991; Frings and Lindemann, 1991) and/or inositol triphosphate-gated calcium channels (Restrepo *et al.*, 1990) leads to depolarization of the olfactory neuron. The specificity of the interactions between different odorants and their receptors mediates excitation of subpopulations of olfactory neurons, thereby encoding the nature and concentration of odorants as distinct patterns of neural activity (Sicard and Holley, 1984). Decoding of these patterns is initiated in the olfactory bulb, the first synaptic relay in the brain, where 100-1000 axons of primary olfactory receptor neurons converge on the dendritic arbor of a single output neuron forming complex synaptic structures, known as "glomeruli."

Since olfaction involves activation of a G-protein regulated adenylyl cyclase (Pace *et al.*, 1985; Sklar *et al.*, 1986; Shirley *et al.*, 1986; Anholt *et al.*, 1987; Anholt, 1988; Jones and Reed, 1989; Anholt and Rivers, 1990; Breer *et al.*, 1990), odorant receptors were postulated to belong to the superfamily of G-protein linked receptors. Pursuing this hypothesis, Buck and Axel (1991) identified from a rat olfactory library a set of cDNAs which encoded a new group of G-protein linked receptors. These receptors showed the superfamily's characteristic seven transmembrane domain motif (O'Dowd *et al.*, 1989) and were expressed only in olfactory tissue. Their expression in olfactory neurons (Ngai *et al.*, 1993b; Raming *et al.*, 1993) and preliminary functional studies (Raming *et al.*, 1993) indicate that they are, in fact, odorant receptors. The intracellular regions of their transmembrane domains are connected via relatively short cytoplasmic loops and they show considerable sequence diversity, especially in their third, fourth and fifth transmembrane domains which, by analogy to other members of the G-protein-linked receptor superfamily, may participate in the formation of the odorant binding site. In mammals the size of this family of odorant receptors is surprisingly large and may be encoded by hundreds or even thousands of genes (Buck and Axel, 1991; Levy *et al.*, 1991). Subsequently, a homologous family of odorant receptors was described in catfish (Ngai *et al.*, 1993a and b) and genes encoding similar odorant receptors were found in the human genome (Selbie *et al.*, 1992). The dazzling number of olfactory receptor genes, the diversity of transduction pathways to which they may be linked and the vast size of the odorant repertoire have hampered progress in the identification of functional odorant receptors. Difficulties in matching defined odorants to their receptors also have limited studies of their ligand specificities. Furthermore, to date no experiments have been reported which

link the molecular interaction between an odorant and its receptor to a defined behavioral response.

The goal of this ARO-sponsored research program is to characterize odorant receptors functionally. We have constructed a chimaeric receptor that contains a putative odorant binding domain of odorant receptor I-15 (Buck and Axel, 1991) linked to the intracellular transduction machinery of the β_2-adrenergic receptor known to interact with adenylate cyclase via G_s. The specificity of this synthetic binding domain can now be studied in a eukaryotic expression system within a familiar pharmacological context, namely the stimulus-dependent generation of cyclic AMP. In addition, we have devised a strategy that employs *P*-element mediated mutagenesis in highly inbred lines of *Drosophila melanogaster* to provide experimental evidence for the notion that the recognition of a given odorant occurs through interactions with multiple odorant receptors similar to the way in which an antigen is recognized by an ensemble of antibodies in a polyclonal antiserum.

B. SUMMARY OF THE MOST IMPORTANT RESULTS

Our studies on the functional characterization of odorant receptors have developed in two directions. One direction is concerned with the characterization of the ligand specificity of a single defined odorant binding domain. The fourth and fifth transmembrane domains and their extracellular linker of the human β_2-adrenergic receptor have been replaced by the equivalent regions of odorant receptor I-15 (Buck and Axel, 1991), thus forming a chimaeric seven transmembrane domain containing G-protein linked receptor that couples a novel odorant binding domain consisting of the I-15 moiety (and perhaps part of the adrenaline binding site of the adrenergic receptor) to the intracellular regions of the β_2-adrenergic receptor that couples ligand binding to the production of cyclic AMP. Expression of this chimaera in eukaryotic cells and screening the expressed construct with an array of odorants is expected to identify a set of odorants that interacts with the ligand binding domain of the synthetic receptor. The binding site can then be characterized pharmacologically and the interactions of odorants with specific amino acid residues in the hybrid receptor can be studied. This information can then be used to characterize the odorant specificity of the parental I-15 receptor, that may not be identical, but is likely to be closely related to that of the chimaera. The second direction of our research program is concerned with the question of how an ensemble of odorant receptors with overlapping ligand specificities interacts to recognize a given odorant. To the best of our knowledge only one experimental system is available to address this issue directly: highly inbred lines of *Drosophila melanogaster* that contain single *P*-element inserts giving rise to mutations at single loci within a co-isogenic genetic background. This system allows a multidisciplinary approach that enables correlation of the expression of identified olfactory genes with behavior of the animal. We have established a highly productive and fruitful collaboration with Dr. Trudy Mackay at the Department of Genetics at North Carolina State University to pursue this direction. A detailed description of these two aspects of our research program follows below.

I. STUDIES OF AN ODORANT RECOGNITION DOMAIN IN A CHIMAERIC RECEPTOR.

a. Construction of a chimaeric receptor

We constructed a cDNA that encodes a chimaeric receptor consisting of the β_2-adrenergic receptor of which the fourth and fifth transmembrane domains and their extracellular linker have been replaced by a cassette that corresponds to the equivalent region of odorant receptor I-15 (Buck and Axel, 1991). The clone encoding the rat odorant receptor I-15 in the pBluescript vector was generously donated by Dr. Linda Buck and a clone encoding the human β_2-adrenergic receptor, also in pBluescript, was donated by Dr. Robert J. Lefkowitz. The region of the odorant receptor that encodes the fourth and fifth transmembrane domains was amplified by PCR using the following 25-mer primers: 5'-TGATCAGTCTGGTGGTGCTGTCCTG-3' and 5'-GTAGAC-AATGATGAGCACAATGGA-3'. The resulting amplification product of approximately 225 bp contains restriction sites for Bcl1 and Acc1. This PCR product was cloned into the T-A cloning vector of Invitrogen and can be released from the vector by double digestion with Bcl1 and Acc1. The β_2-adrenergic receptor clone contains restriction sites for Bcl1 at nucleotide positions 1718 and 1901 and an Acc1 restriction site at nucleotide 1916. Thus a 192 bp fragment can be excised from this cDNA that encodes the fourth and fifth transmembrane domains of this receptor and exchange of this region by the Bcl1/Acc1 digested odorant receptor PCR fragment described above would provide an intact open reading frame that encodes the desired chimaeric receptor. In order to be able to make this construct we first had to subclone the cDNA encoding the β_2-adrenergic receptor into pGEM7Zf(+), a vector that contains neither a Bcl1 nor an Acc1 restriction site. The cDNA that encodes the β_2-adrenergic receptor subcloned into pGEM7Zf(+) was digested with Bcl1 and Acc1 and the Bcl1/Acc1 released insert from the T-A cloning vector containing the PCR fragment that encodes the odorant binding domain was ligated into the linearized pGEM7Zf+ construct. To verify that the chimaera was formed correctly in frame the regions spanning both ligation sites were sequenced using the dideoxynucleotide chain termination method (Sanger *et al.*, 1977). The corresponding amino acid sequence of the original β_2-adrenergic receptor in these regions are:

...LLTKNARVI---ILMVW.....PLVIMVFV---YSRVFQ...

(--- indicates the positions of ligation sites)

The corresponding amino acid sequences of odorant receptor I-15 in this region are as follows:

...IMSPKLCV---SLVVS.....PFVLIIVS---YARVVA...

The cDNA for the chimaeric receptor which we constructed encodes the amino acid sequences:

...LLTKNKARVI---*SLVVS*.....*PFVLIIV*---YSRVFQ....

b. Functional characterization of the chimaeric receptor

To functionally characterize the chimaeric receptor encoded by our cDNA construct we subcloned the construct into the eukaryotic expression vector pCEP4. This is a 10.4 kb Epstein Barr virus derived vector that in primate cells replicates extrachromosomally to high copy number (app. 30/cell). We have cloned our chimaeric cDNA construct and the cDNA encoding the parental β_2-adrenergic receptor between the Xho1 and BamH1 sites of the multiple cloning site in the correct

orientation to allow expression of the chimaeric receptor under the control of a strong cytomegalovirus promoter. pCEP4 carries the β-lactamase gene that confers ampicillin resistance for maintenance in E. coli. We are presently establishing optimal conditions for introduction of the pCEP4 constructs in HeLa cells via electroporatio · Since the pCEP4 vector confers hygromycin B resistance to the cells, we will grow transfectants in the presence of 0.5 mg/ml hygromycin B to select and maintain stable transfectants. We anticipate to have these cells in hand within the next month and will begin screening expressed receptors with our library of odorants to identify those odorants that lead to the generation of cyclic AMP. We will then be in a position to investigate the binding specificity of part of an isolated odorant binding domain within a familiar, well-characterized pharmacological context. Ultimately, this line of research will tie in with our work on Drosophila, described below, since we may be able to introduce via a transposable element derived vector our hybrid construct into germ line cells of *Drosophila melanogaster*, thus creating a line of flies that may display a behavioral response to a perhaps previously unrecognized odorant. This would be the first time that a synthetic odorant receptor would be introduced into an organism to permanently alter that organism's chemosensory perception.

II. IDENTIFICATION OF OLFACTORY RECEPTOR GENES BY SINGLE *P*-ELEMENT MUTAGENESIS OF INBRED *DROSOPHILA MELANOGASTER.*

In collaboration with the laboratory of Dr. Trudy F. C. Mackay at the Department of Genetics at North Carolina State University we decided to exploit the relative simplicity of the olfactory system of *Drosophila melanogaster* and its accessibility to genetic and biochemical experimentation to characterize the response to one well-defined odorant, benzaldehyde, at the behavioral, genetic and molecular level. Highly inbred lines of flies that carry single randomly inserted *P*-element constructs (*P[lAr]B*) in their genome are screened to identify mutants deficient in the avoidance response to one well-defined odorant, benzaldehyde. *P*-element-tagged genomic DNA sequences can then be isolated from behavioral mutants in which the *P*-element-linked *lacZ* reporter gene is expressed in chemosensory structures. Analysis of these sequences will lead to the identification of gene products that are essential for the odorant elicited behavioral response. Such gene products are likely to include odorant receptors.

Drosophila melanogaster presents an ideal model system for multidisciplinary studies on odor recognition. Fruit flies have a relatively simple olfactory system consisting of approximately 10^3 receptor neurons (as compared to 10^8 in humans) located in the third segment of the antenna and projecting to about 35 glomeruli in each antennal lobe (Stocker *et al.*, 1990; Carlson, 1991). In addition chemosensory information can be received via the maxillary palps. In larvae the antennal organ is believed to mediate odor recognition and contains approximately 20 neurons of which the cell bodies are located in the antennal ganglion (Kankel *et al.*, 1980; Singh and Singh, 1984). The reduced complexity of its olfactory system suggests that the odor repertoire to which Drosophila responds is relatively restricted. Furthermore, powerful genetic approaches exist for studies on Drosophila and reproducible behavioral assays to quantitate olfactory responses can be established with relative ease.

Studies on olfactory behavior of Drosophila were pioneered by elegant experiments by Siddiqi and co-workers who demonstrated that a classical Y-maze paradigm could be utilized to

isolate a number of olfactory mutants (Rodrigues and Siddiqi, 1978; Ayyub *et al.*, 1990). Subsequently, the olfactory behavior of Drosophila has also been exploited for studies on conditioned learning and has resulted in the identification of the mutants, *dunce* and *rutabaga*, which, respectively, represent defects in a cyclic AMP phosphodiesterase (Chen *et al.*, 1986) and a calcium-calmodulin sensitive adenylyl cyclase (Levin *et al.*, 1992) both expressed in mushroom bodies (Nighorn *et al.*, 1991; Han *et al.*, 1992) implying cyclic AMP as a central modulator in learning and memory. Additional olfactory mutants which have been identified include *smellblind*, a general anosmic mutant, localized to the X-chromosome (Aceves-Piña and Quinn, 1979), *pentagon*, an X-linked mutant unresponsive to benzaldehyde (Helfand and Carlson, 1989), and several mutants defective in an odorant-induced behavioral jump response (McKenna *et al.*, 1989). Of special interest is the demonstration that the *retinal degeneration B (rdgB)* gene encodes a protein essential both for normal visual and olfactory physiology in Drosophila, suggesting that in insects, like in vertebrates, parallels exist between pathways for visual and olfactory transduction (Woodard *et al.*, 1992). In addition to olfactory mutants, several gustatory mutants showing abnormal responses to quinine or NaCl have also been reported (Tompkins *et al.*, 1979). These studies demonstrate that chemosensory behavior in Drosophila can be readily assayed and that a host of mutants can be isolated. Thus far, however, most of these mutations were localized to the X-chromosome and none of these mutations has led to the identification of an odorant receptor. Furthermore, since mutations were induced chemically, cloning and sequencing of the affected gene products is laborious. In addition, behavioral assays were designed primarily to detect large qualitative defects in behavioral responses to odorants rather than more subtle partial aberrations. The latter would be the expected phenotype if a given odorant reacts with several low-affinity receptors of broad specificity. In this case, a defect in a single receptor would attenuate, but not necessarily eliminate the detection of a specific odorant.

Several approaches could be employed to identify odorant receptors in Drosophila. Screening a genomic library by hybridization at low stringency with cDNAs encoding mammalian odorant receptors may identify homologous sequences in the fly. However, since odorant receptors are members of the large superfamily of G-protein-linked receptors, the nature of receptor proteins of unknown ligand specificity obtained by this approach would be doubtful. Previously, Carlson and his colleagues screened 6,500 Drosophila lines, each containing a transposable element construct inserted at a unique locus (Riesgo-Escovar *et al.*, 1992). They postulated that if the insertion site for this element is near an olfactory gene, the tissue-specific enhancer for that gene will drive expression of β-galactosidase, contained as a reporter gene in the *P*-element construct, in olfactory structures. Indeed, 12 lines were identified which showed reporter gene expression mainly in larval and adult olfactory organs (Riesgo-Escovar *et al.*, 1992). Although these results are encouraging, the lack of previous phenotypic characterization of these lines renders it difficult to relate *lacZ* expression in olfactory tissues back to defined olfactory behavior of the fly. Therefore, we decided first to identify flies defective in their response to a well defined odorant and then to select for further analysis those lines that show expression of β-galactosidase in the major olfactory tissues of adult flies, i.e. the third antennal segment and maxillary palps, and the antennal organs of larvae.

We postulate that detection of a given odorant, such as benzaldehyde, involves interactions between the odorant and multiple members of the odorant receptor family similar to the recognition of an antigen by a spectrum of antibodies in a polyclonal

antiserum. We are, therefore, analyzing the olfactory behavior of Drosophila as a quantitative trait. Identifying individual loci contributing to variation in a quantitative trait, such as the recognition of the odorant benzaldehyde, is generally not possible in random breeding populations because the effects of any single locus affecting the trait are obscured by the effects of the other segregating loci and the environmental variation. The most common procedure for identifying quantitative trait loci is to cross highly selected or inbred lines which differ widely for the character of interest and to detect the individual loci by linkage to polymorphic morphological, isozyme or molecular markers in F2 or backcross progeny. Application of this method results in estimates of map positions and effects of quantitative trait loci, but considerable further effort is required to determine the molecular nature of the identified quantitative trait loci. However, in Drosophila it is possible to identify and map quantitative trait loci using *P*-element insertional mutagenesis (Lai and Mackay, 1990 and 1993; Mackay *et al.*, 1992). Loci affecting the trait of interest are identified by a detectable change in mean trait value of a highly inbred line into which the *P*-element has inserted. The location of the inserted sequence (and hence the quantitative trait locus) can be mapped precisely by *in situ* hybridization to polytene chromosomes, and the inserted sequence tags the gene for cloning (Bingham *et al.*, 1981). All loci affecting the trait which are susceptible to insertional mutagenesis and which mutate to alleles with detectably altered phenotypes can in principle be discovered by this method. This approach has been used to identify loci affecting numbers of sensory hairs, a standard quantitative trait in Drosophila (Mackay *et al.*, 1992), and we are currently applying it to identify and clone loci affecting odor recognition.

We currently have at our disposal approximately 400 *X*, second and third chromosome lines derived in a highly inbred background, each containing a single insertion of the *P*-element-derived *P*[lArB] construct (Bellen *et al.*, 1989; Fig. 1). Based on avoidance of the odorant benzaldehyde we have established a simple and reliable behavioral assay that allows us to quantitate benzaldehyde-induced avoidance behavior and to detect insert lines with aberrant behavior. These deviant lines are then examined for tissue-specific expression of the *lac-Z* reporter gene. Lines with expression confined to larval and adult olfactory tissues contain inserts at olfactory loci. Correlation of the mutant phenotype with the presence of the transposable element can be confirmed by testing wild-type revertant lines from which the *P*[lArB] element has excised. Genomic DNA of the olfactory locus can be recovered from the insert line by plasmid rescue. We are currently analyzing such genomic clones to identify nucleotide sequences that encode olfactory proteins essential for mediating the avoidance response to benzaldehyde. It is likely that odorant receptors, like those already identified in several species, including rat (Buck and Axel, 1991; Levy *et al.*, 1991; Raming *et al.*, 1993), human (Selbie *et al.*, 1992) and catfish (Ngai *et al.*, 1993a and b), will be among this group of proteins. Such putative odorant receptors, expected to recognize benzaldehyde, will then be expressed and functionally characterized in Sf9 insect cells using a baculovirus expression system.

a. Construction and analysis of mutant lines

P-element mutagenesis is routinely used in Dr. Mackay's laboratory to construct *X*, second, and third chromosome lines which each contain a single stable *P*-element construct integrated into a highly inbred host background initially devoid of *P*-elements. A modification

of the "jumpstart" scheme proposed by Robertson *et al.* (1988) and Cooley *et al.* (1988) is used for insertional mutagenesis. The *P[ry⁺Δ2-3](99B)* element is used as a stable genomic source of *P* transposase to mobilize the *P*[lArB] plasmid constructed in the laboratory of W. Gehring (Bellen *et al.*, 1989). The *P*[lArB] plasmid is a modified *pUChsneo* plasmid containing the *P-lacZ-hsp 70* fusion gene, the *Adh⁺* and *ry⁺* genes, and the Bluescript plasmid vector (Bellen *et al.*, 1989; Fig. 1), facilitating identification of transformants in an *ry⁻* background and subsequent cloning of genomic sequences adjacent to the site of insertion. Although *P*-element-induced mutation rates vary across loci (Kidwell, 1986), *P*-element mutagenesis has proven an effective procedure for recovering mutations at most major loci studied to date. The scheme shown in Fig. 2 is a less labor-intensive alternative to transformation by microinjection of *P*-elements (Spradling and Rubin, 1982), avoids all the problems of hybrid dysgenesis-mediated *P*-element transposition (Mackay, 1986, 1987 and 1989; Lai and Mackay, 1990) and has proven to be effective at inducing mutations at quantitative trait loci (Mackay *et al.*, 1992).

The following Drosophila strains have previously been constructed in Dr. Mackay's laboratory. Genetic symbols not otherwise explained are in Lindsley and Zimm (1992). (i) Inbred Samarkand (*Sam*). This standard inbred stock has been maintained by Dr. Mackay for over 140 generations by continuous full-sib inbreeding. It is free of *P*-elements. (ii) *Sam1;Sam2;ry⁵⁰⁶*. A single *ry⁵⁰⁶* third chromosome was made isogenic and substituted into the inbred *Sam* genetic background. All *ry⁵⁰⁶* chromosomes in the strains described below are replicates of this single chromosome. (iii) *C(1)DX,y w f/Sam1;Sam2;ry⁵⁰⁶*. The *C(1)DX* chromosome is abbreviated below as *XX*. (iv) *Sam1;Sam2;ry⁵⁰⁶Sb P[ry⁺Δ2-3](99B)/TM6,Ubx (Sam1;Sam2;SbΔ2-3/Ubx)*. (v) *Sam1;CyOP[lArB]/Pm;ry⁵⁰⁶*. The *P*[lArB] plasmid is inserted on the *CyO* balancer chromosome, abbreviated *CyO-P*. (vi) *Sam1;Cy/Sp;ry⁵⁰⁶*. The *Cy* marker is on the *SM5* balancer chromosome.(vii) *Sam1;Cy/Sp;TM3,Sb ry^RK/ry⁵⁰⁶*. The third chromosome balancer has a recessive X-ray induced *ry* allele (Karess and Rubin, 1984) to allow scoring for *ry⁺* transformants. It is abbreviated *Sb ry^RK*. (viii) *Basc;Sam2;ry⁵⁰⁶*. (ix) *Sam1;Sam2;Sb ry^RK/Tb*. The *Tb* marker is on the *TM6B* balancer chromosome.

To generate insertions of *P*[lArB] on the *X*, second and third chromosomes of the isogenic *Sam1;Sam2;ry⁵⁰⁶* strain crosses are performed as indicated in Fig. 2. The first cross mobilizes the *P*-element. Subsequent crosses fix the *P*-element in place generating stable mutant lines. Thus, this crossing scheme allows the construction of *X*, second, and third chromosome lines that differ only in the position of the *P*[lArB] insert in a common isogenic background. Presence of the *P*[lArB] insert is evident from the red eye color resulting from the *ry⁺* gene. Its location on the second or third chromosome can be determined from the proportion of red-eyed (ry^+) *Cy;Sb* flies (100% for chromosome 2 inserts; 50% for chromosome 3 inserts).

b. Behavioral Assay

To enable reliable screening of hundreds of *P*-insert lines, we have developed a rapid, simple and highly reproducible behavioral assay. We considered Y-maze assays used previously (Rodrigues and Siddiqui, 1978; Ayyub *et al.*, 1990) too complex and the jump response (McKenna *et al.*, 1989) was, in our hands, too variable. We discovered that responses of Drosophila to odorants can be quantitated with great efficiency and reproducibility using a simple "dipstick" method.

Flies of the strain to be tested are collected 4-7 days post-eclosion, and stored in single-sex groups of 5 individuals in clear plastic Drosophila culture vials without medium for 3-6 hours prior to testing. The 2.5 x 9.5 cm vials are marked with two lines 3 cm and 6 cm from the bottom. A Q-tip dipped in distilled water or odorant is inserted inside the tube so the tip lines up against the 6-cm mark, protruding approximately 1 cm below the cotton wool plug. Since the flies are positively geotactic, the vial is placed on its side to start the assay, always in the same orientation. The flies are given 15 seconds to recover from the disturbance during the insertion of the Q-tip, then ten counts of the number of flies in the bottom compartment of the tube demarcated by the 3-cm line are taken at 5-second intervals, starting with the 15-second time point. Thus, the total duration of the assay following introduction of the Q-tip is one minute. Two replicate assays of each line are done on different days, and each replicate consists of five males and five females, tested separately. The behavior of each line is quantified as the "avoidance score", which is calculated as the number of flies in the bottom sector of the vial, averaged over the 10 measurements, two sexes and two replicates. Thus, the avoidance score for each line is based on 40 observations for each chemosensory stimulus during the initial screening. A score of 2.5 would indicate the flies are indifferent to the chemosensory stimulus, spending as much time near its source as away from it; scores significantly greater than 2.5 indicate they are repelled by the stimulus and scores significantly less than 2.5 indicate the stimulus attracts them. Statistical analyses that evaluate the reproducibility and significance of correlations between parameters in relation to the sample size are described in detail below and indicate that our behavioral assay yields highly reproducible measurements.

In pilot experiments, we assessed the responses of a sample of wild-type random-bred and inbred strains to a large number of odorants, including but not limited to: benzaldehyde, isoamylacetate, ethanol, acetic acid, propionic acid, menthone, eugenol, 3-isobutyl-2-methoxypyrazine, citralva, limonene, bergamot, and lavender. Flies were tested both individually and in single-sex groups of 5 or 10 and were found to behave independently. Most of the odorants tested gave mean responses close to 2.5; i.e., the flies were indifferent to them under the test conditions. However, we found that flies reliably exhibited a strong and reproducible avoidance response to undiluted benzaldehyde, with virtually all animals congregating at the bottom of the tube by the end of the assay. Some other odorants eliciting avoidance responses proved toxic to the animals. Benzaldehyde, however, did not affect survival of the flies or induce desensitization for exposure periods as long as 30 minutes. For these reasons and since previously other investigators have also used this odorant (McKenna *et al.*, 1989; Ayer and Carlson, 1992; Hefland and Carlson, 1989), we decided to use benzaldehyde as the olfactory stimulus for our studies. After the brief period of starvation prior to the assay, the flies are attracted to Q-tips dipped in distilled water. Thus, each line tested for response to benzaldehyde was first assayed for response to distilled water, allowing us to identify lines that are generally non-responsive or non-motile as well as those that are specifically aberrant in their response to benzaldehyde.

c. Preliminary screen of *P*-insert lines

To verify the feasibility of our experimental strategy, we assayed the olfactory behavior of 82 second and 120 third chromosome lines, each containing a single homozygous *P*[lArB] insert in a highly inbred host strain genetic background, and 20 isogenic control lines of the host

strain with no *P*-element inserts. As described above, two replicate assays were done for each line, and each replicate monitored the response of 5 males and 5 females (separately) to distilled water and to benzaldehyde.

Figure 3 illustrates the mean avoidance responses of the control lines and the homozygous second chromosome *P*-insert lines to distilled water and to benzaldehyde, averaged over the 10 observations per replicate, both sexes and the two replicate assays. For the control lines, the population mean response (± standard error) to distilled water was 1.60 ± 0.08 and to benzaldehyde was 3.94 ± 0.06, demonstrating the clear attraction to the former and avoidance of the latter under the test conditions. For the *P*-insert lines, the population mean response to distilled water was 1.53 ± 0.06 and to benzaldehyde was 3.59 ± 0.05. The lower mean response of the *P*-insert population to benzaldehyde is due to seven lines with responses to benzaldehyde that overlap the water responses. These seven lines, with mean responses of 3.0 or less, are putative olfactory mutants.

Figure 4 shows the reponses of control and homozygous *P*-insert lines to water and benzaldehyde over the time course of the assay, averaged over all lines, sexes and replicates. The response to water is constant throughout the assay, and does not differ for control and *P*-insert lines. Initially flies of all lines tend to avoid benzaldehyde, with approximately 60% of the flies of both control and insert lines (corresponding to a mean avoidance score of 3.0) in the bottom sector of the vial at the start of the assay, i.e. 15 seconds after insertion of the Q-tip. By the last measurement approximately 85% of the flies are distributed away from the odor source. The response to benzaldehyde of the seven putative olfactory mutant lines is shown separately in the bottom panel of Figure 4. Flies from these lines tend to avoid benzaldehyde, but their responses are weak and appear delayed.

These data were quantified further by four-way factorial analyses of variance of the control and *P*-insert lines separately, considering time, sex, and odor as fixed effects and line as a random effect. These analyses are presented in Table 1. As noted from visual inspection of data from both control and *P*-insert lines, the overall effect of time of measurement is highly significant (the benzaldehyde avoidance response is stronger at the end of the assay; compare the MS values for control and *P*-insert lines on the first line of Table 1), as is the main effect of odor (flies avoid benzaldehyde and are attracted to distilled water; compare the MS values for control and *P*-insert lines on the third line of Table 1). There is also a highly significant time x odor interaction (the response curve for distilled water is flat, but that for benzaldehyde increases over the assay period; observe the MS values on the fifth line of Table 1). The sex x odor interaction term is highly significant for the *P*-insert lines but non-significant for the control lines; this indicates *P*-element-induced mutational variation in sex dimorphism for odor response averaged over all lines. There is significant variation among control and *P*-insert lines, but the estimate of this variance component is nearly three times greater for the *P*-insert lines, demonstrating significant *P*-element-induced mutational variation for odor response (in Table 1 compare the σ^2 values of control lines to those of *P*-insert lines). There is also evidence for variation among *P*-insert lines in sex dimorphism of response (sex x line interaction), and the increased line x odor variance component of the *P*-insert lines quantifies the observation that response to benzaldehyde varies in these lines more than response to water. Finally, the excellent agreement between estimates of variance between replicates (indicated on the last line in Table 1 as error variance) of both control and *P*-insert lines attests to the reproducibility of the assay.

d. Enhancer-trap analysis of putative olfactory mutant lines

The *lacZ* gene of the *P*[lArB] construct has a weak promotor but lacks an enhancer. If the construct inserts near an enhancer element, expression of β-galactosidase will be driven by the gene to which the enhancer is linked. If the inserts in the seven lines with low benzaldehyde avoidance scores are indeed in olfactory loci, then β-galactosidase should be expressed in olfactory tissues (primarily the third antennal segment and maxillary palps of adults, and antennal organs in larvae).

β-galactosidase expression was examined in adults and larvae of the seven lines that showed weak responses to benzaldehyde, as well as in six *P*-insert lines with wild-type responses to benzaldehyde. Adult males and females (at least two of each sex) were decapitated, and a dorsal incision was made in the heads to allow penetration of reagents. Heads and bodies were stained in microtiter plates for β-galactosidase activity according to the procedure given by Ashburner (1989). Following the staining, they were dissected and examined under a Zeiss Axioplan microscope. Third instar larvae were dissected into two parts prior to staining by cutting at the fifth segment, then stained and examined for the pattern of expression of β-galactosidase following the same procedure as for adults.

Tissue-specific expression patterns in adults and larvae of the *P*-insert lines with low and with wild type benzaldehyde avoidance scores are presented in Table 2. It should be noted that highly localized staining in only a few cells in adults or larvae may have gone undetected (indicated as "none detected" in the table). We will, therefore, repeat the experiment shown in Table 2 several times using an anti-β-galactosidase antibody for higher resolution to determine whether lack of detection of β-galactosidase is an artifact due to insufficient penetration of reagents, whether it is due to highly localized expression of the reporter gene or whether the gene tagged by the *P*-element is not expressed in larvae. Thus far, five of the seven lines with low benzaldehyde avoidance scores displayed detectable staining in the third antennal segment of adults. In three of the lines expression in adults appears limited to this organ. Two lines show staining in the second antennal segment and the maxillary palps as well. One example of such a line, line *P(2)173*, is presented in Figure 5. It shows intense staining at the tips of the maxillary palps and of the third and second antennal segments. Staining appears to be more intense in the proximal region of the third antennal segment than in the distal region and is restricted to the anterior tips of the maxillary palps. This pattern of differential expression is reminiscent of the "expression zones" of vertebrate odorant receptors revealed by *in situ* hybridization (Vassar *et al.*, 1993; Ressler *et al.*, 1993). It is also of interest to note that line *P(2)173* displays a normal avoidance response to menthone. Thus, this line shows the differential expression pattern and specificity in responsiveness to odorants expected of a mutant line defective in the expression of an odorant receptor. One of the two lines with a low benzaldehyde avoidance score, line *P(2)131*, did not reveal detectable staining in adult flies, but showed highly localized and intense staining in larval antennal organs (Fig. 6). In contrast to the staining patterns of lines with low benzaldehyde avoidance scores, only one of the six lines with wild type benzaldehyde avoidance scores exhibited expression of β-galactosidase in the third antennal segment, and in this line the staining was weak. Thus, our behavioral screen appears to have succeeded in identifying lines with inserts affecting olfactory loci. This observation confirms the validity of our behavioral assay and our statistical analyses. We have already begun to rescue

P[lArB]-tagged genes as pBluescript plasmids from seven mutant lines and we anticipate that we will have molecular information regarding the identity of approximately a dozen olfactory genes, including some recently identified third chromosome genes, within the next several months.

C. LIST OF PUBLICATIONS

Anholt, R. R. H. (1993) Molecular neurobiology of olfaction. Critical Rev. Neurobiol. **7**: 1-22.

Anholt, R. R. H. (1994) Signal integration in the nervous system: Adenylate cyclases as molecular coincidence detectors. Trends Neurosci., **17**: 37-41.

Anholt, R. R. H., Lyman, R. F. and Mackay, T. F. C. (1994) Identification of olfactory genes by single *P*-element mutagenesis of inbred *Drosophila melanogaster*. Sixteenth Annual Meeting of the Association for Chemoreception Sciences, in press.

D. LIST OF SCIENTIFIC PERSONNEL

Robert R. H. Anholt, Ph. D.	Principal Investigator
Laurel Evers	Research Technician
Nancy Andon	Research Technician
Steven Lesser	Graduate Rotation Student
Trudy F. C. Mackay, Ph.D.	Collaborator
Richard F. Lyman, Ph.D.	Postdoctoral Fellow (with Dr. Mackay)

5. REPORT OF INVENTIONS

There were no inventions during the period covered by this report.

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Table 1. Analyses of variance of odor responses of control and *P* element insert lines.

	Control Lines			*P*-Insert Lines		
Source of Variation	df	MS	σ^2	df	MS	σ^2
Time	9	8.87^{***}		9	33.79^{***}	
Sex	1	46.24^{***}		1	15.12^{**}	
Odor	1	2199.61^{***}		1	6958.52^{***}	
Sex x Time	9	0.69^{ns}		9	0.26^{ns}	
Odor x Time	9	5.29^{***}		9	29.27^{***}	
Sex x Odor	1	0.72^{ns}		1	69.07^{***}	
Sex x Odor x Time	9	0.85^{ns}		9	0.74^{ns}	
Line	19	5.13^{***}	0.052	80	13.49^{***}	0.157
Line x Time	171	0.56^{ns}	(-)	720	0.57^{ns}	(-)
Sex x Line	19	2.15^{***}	0.030	80	5.96^{***}	0.125
Line x Odor	19	3.02^{***}	0.052	80	6.54^{***}	0.140
Sex x Line x Time	171	0.61^{ns}	(-)	720	0.55^{ns}	(-)
Line x Odor x Time	171	0.51^{ns}	(-)	720	0.49^{ns}	(-)
Sex x Line x Odor	19	4.22^{***}	0.164	80	6.14^{***}	0.260
Sex x Line x Odor x Time	171	0.61^{ns}	(-)	720	0.60^{ns}	(-)
Error	800	0.94	0.944	3240	0.94	0.942

***: $P < 0.001$; **: $0.001 < P < 0.01$; ^{ns}: $P > 0.05$

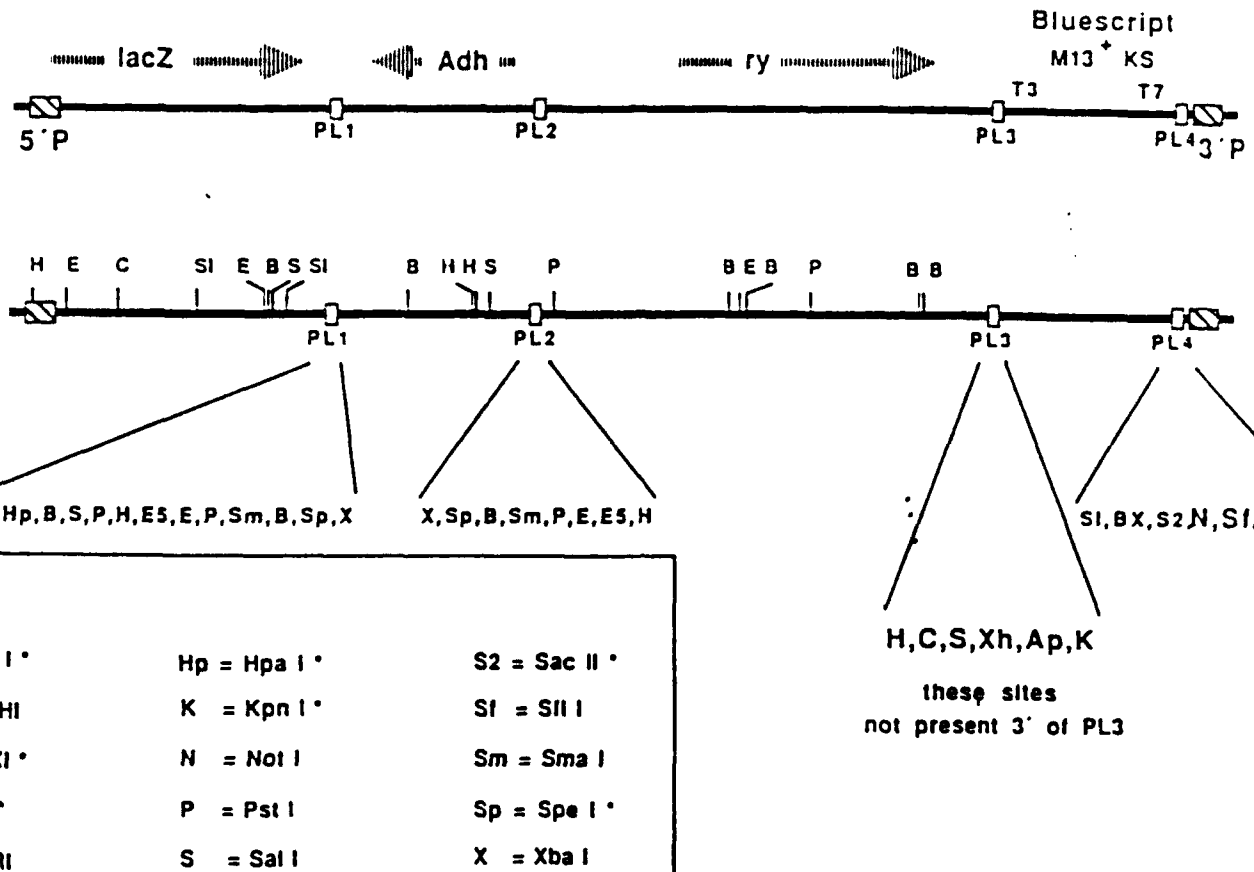
Responses to distilled water and benzaldehyde, as described in the text, were collected for 20 individuals of each line.

df designates the degrees of freedom, MS the mean squares and σ^2 the variance component.

(Note: One of the Chromosome 2 insert lines assayed was homozygous male-lethal; this line was excluded from the analysis of variance to completely balance the design)

Table 2. Tissue-specific β-galactosidase expression in *P*-insert lines.

Line	Tissues Stained: Adults	Tissues Stained: Larvae
	Weak Benzaldehyde Response Lines	
P(2)36	3rd antennal segment (diffuse)	none detected
P(2)68	3rd antennal segment (diffuse)	none detected
P(2)89	3rd antennal segment	salivary gland, brain (light), testis (dark staining in larvae and pharate, but not in mature adults)
P(2)128	none detected	none detected
P(2)131	none detected	antennal organs
P(2)173	3rd antennal segment (intense); 2nd antennal segment; maxillary palps; cells in foregut; wing veins; tarsi and around joints of legs	midgut imaginal islands
P(2)299	3rd antennal segment; 2nd antennal segment; maxillary palps	b r a i n ? o v a r i e s ?
	Wild Type Benzaldehyde Response Lines	
P(2)13	none detected	dorsal prothoracic disc
P(2)57	brain	brain
P(2)138	general epidermal	none detected
P(2)219	head, not antennae	midgut imaginal islands; salivary glands
P(2)342	3rd antennal segment (weak)	none detected
P(2)240	none detected	dorsal prothoracic disc



LEGEND

Ap = Apa I *	Hp = Hpa I *	S2 = Sac II *
B = Bam HI	K = Kpn I *	Sf = Sfi I
BX = Bst XI *	N = Not I	Sm = Sma I
C = Cla I *	P = Pst I	Sp = Spe I *
E = Eco RI	S = Sal I	X = Xba I
E5 = Eco RV *	SI = Sac I *	Xh = Xho I *
H = Hind III	* = all sites have not been mapped	

Figure 1: Schematic representation of the *P*[lArB] construct used for insertional mutagenesis in *Drosophila*. The construct consists of a 31 bp inverted repeat necessary for transposition and insertion, a *lacZ* gene, a reversely oriented *Adh* gene, and a *ry* gene. The latter encodes xanthine dehydrogenase, a visible selectable marker, which rescues the *rosy* phenotype of the inbred *ry*⁻ host strain allowing flies with red eyes to be identified as carrying the *P*[lArB] construct. *P*-element-tagged genes can be retrieved in the pBluescript vector by digestion of genomic DNA with restriction endonucleases that cut at the PL3 polylinker site.

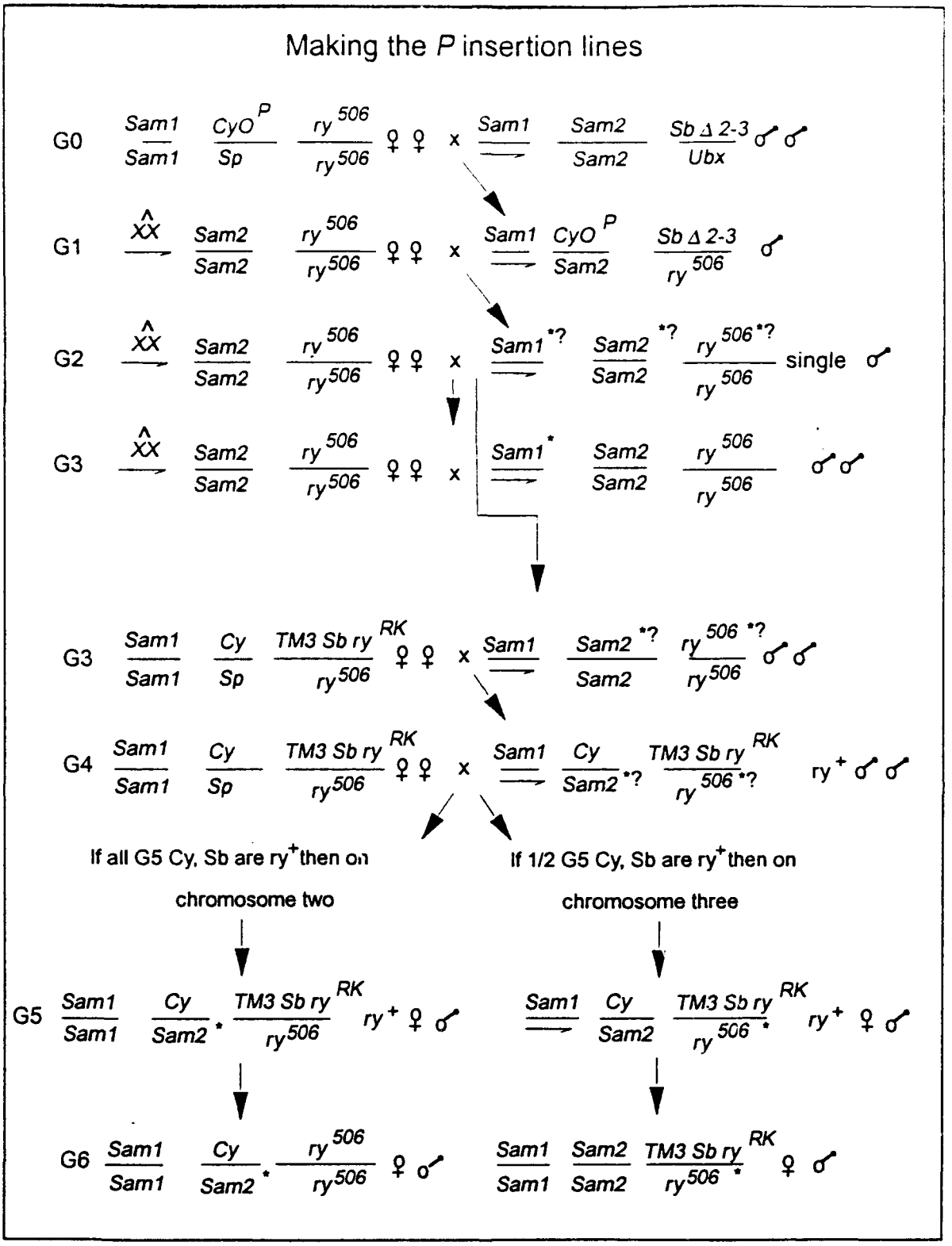


Figure 2: Construction of P-insertion lines.

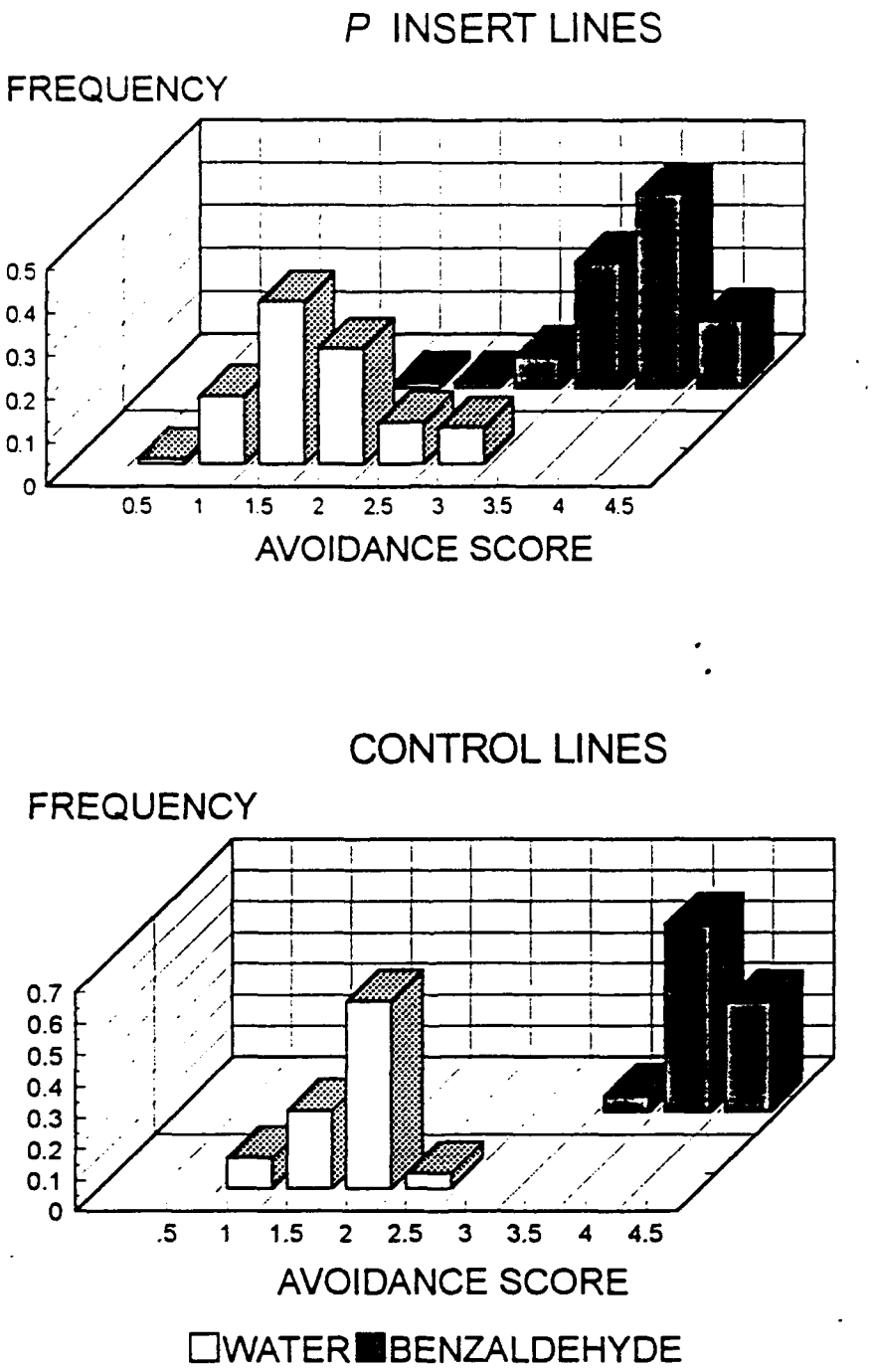


Figure 3: Distribution of avoidance scores for water and benzaldehyde in a population of 82 second chromosome *P*-insert lines (upper graph) and 20 isogenic *P*-element free controls (lower graph). The upper graph reveals seven lines with benzaldehyde avoidance scores of 3.0 or less, overlapping with the distribution of avoidance scores for water. These lines are further characterized in Figure 4 and Table 1.

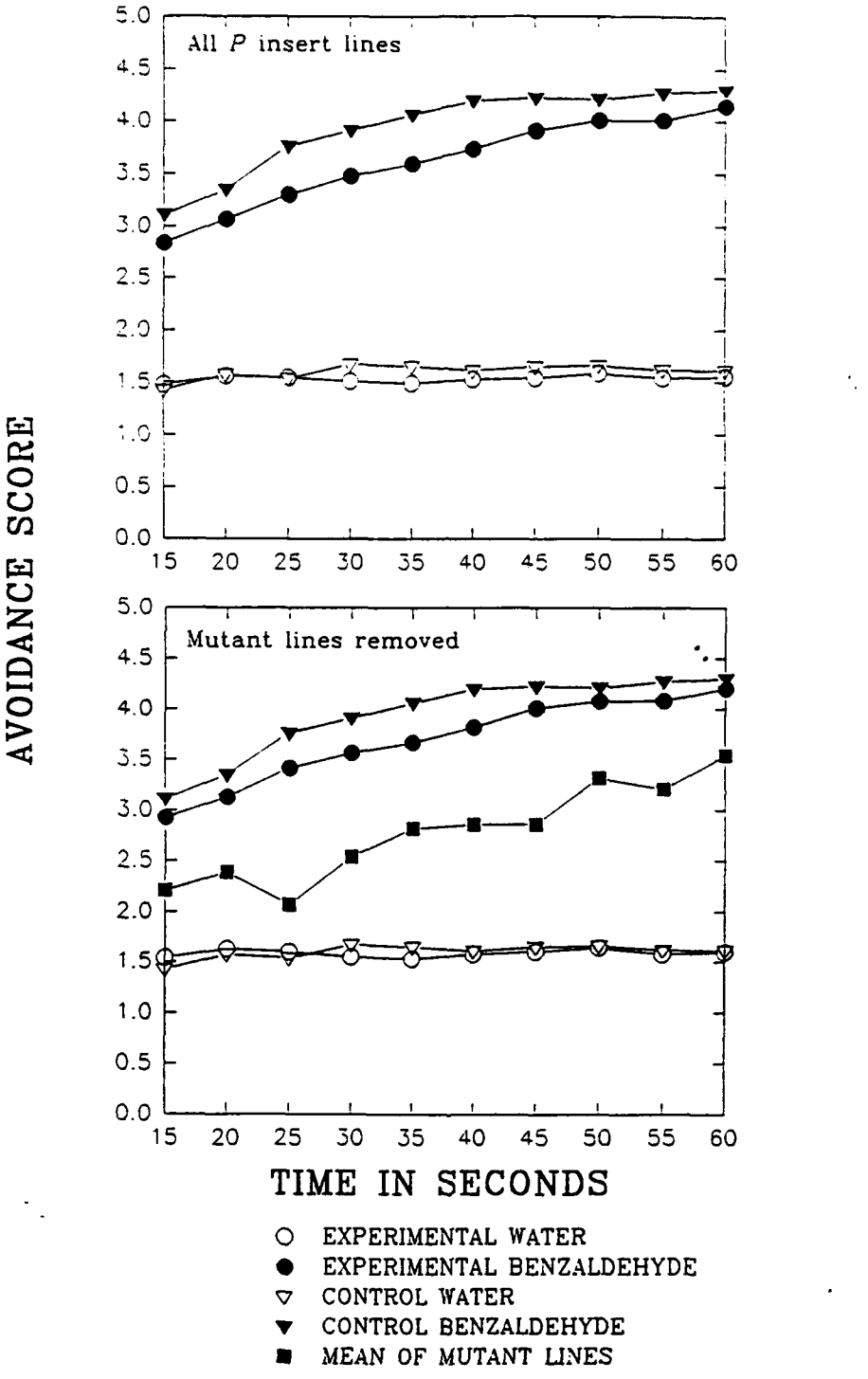


Figure 4: Time course of the avoidance response to benzaldehyde. The kinetics of the response to water (open symbols) or benzaldehyde (closed symbols) was averaged over all lines, sexes and replicates. The population-averaged time course of the response to benzaldehyde of the *82 P*-insert lines is slightly slower than that of the 20 isogenic *P*-element free controls, mainly due to the presence of seven lines with low benzaldehyde avoidance scores (see also Fig. 3). The lower panel shows separately the time course of the benzaldehyde avoidance response averaged over these seven lines (solid squares) and of the remaining 75 *P*-insert lines (solid circles) .

Figure 5: Expression of β-galactosidase activity in antennae (top) and maxillary palps (bottom) of line *P(2)173*. Line *P(2)173* is one of seven lines with low benzaldehyde avoidance scores detected by our behavioral assay (see Fig. 3 and 4, Table 2). Expression of β-galactosidase is evident in the second and third antennal segments and at the tips of the maxillary palps, structures show to participate in odor recognition in *Drosophila* (Ayer and Carlson, 1992). Although staining of the third antennal segment is prominent throughout, it is particularly intense in the proximal region. Bars represent 0.1 mm.

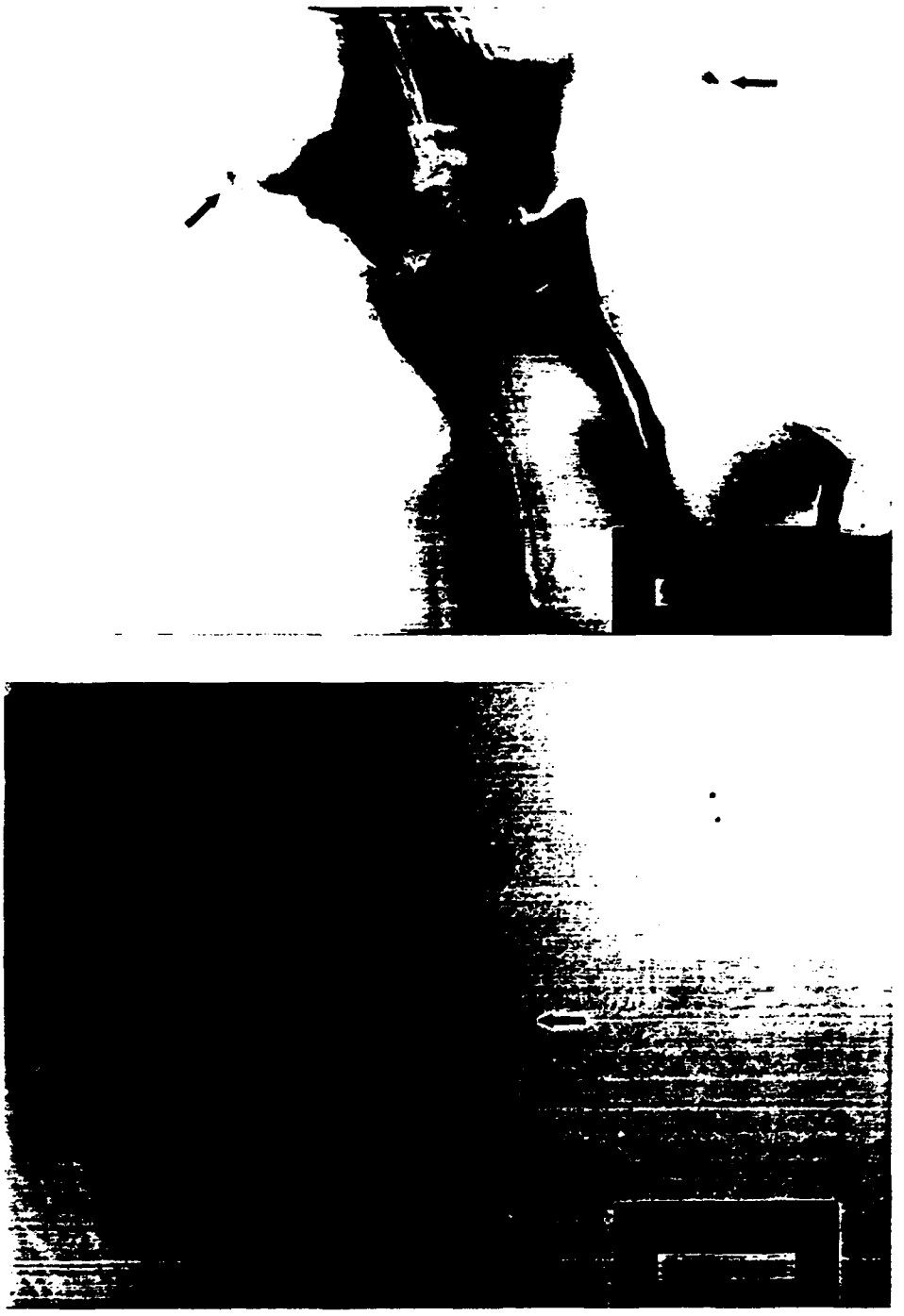


Figure 6: Expression of β-galactosidase activity in larval antennal organs of line *P(2)131*. Line *P(2)131* is one of seven lines with low benzaldehyde avoidance scores detected by our behavioral assay (see Fig. 3 and 4, and Table 2). Expression of β-galactosidase was not detected in adult antennae, but highly localized staining is evident in larval antennal organs (arrows). The bottom panel is a higher magnification view of the top panel. Bars represent 100 μm and 50 μm in the top and bottom panels, respectively.